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Identification of Point Mutations in the Steroid Sulfatase Gene of Three Patients with X-linked Ichthyosis

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Summary

X-linked ichthyosis (XLI) is an inborn error of metabolism caused by steroid sulfatase (STS) deficiency. In more than 80% of XLI patients the enzyme deficiency is due to large deletions involving the entire STS gene and flanking sequences. However, some patients with the classical XLI phenotype and complete STS deficiency do not show any detectable deletions by Southern blot analysis using full-length STS cDNA as a probe. We have studied five unrelated patients who are such "nondeletion" mutants. Western blot analysis using anti-STS antibodies was performed on patients' fibroblast extracts and revealed absence of cross-reacting material. First-strand cDNA synthesis by reverse transcription from patients' RNA isolated from cultured fibroblasts and PCR amplification of overlapping segments of the entire STS polypeptide coding region were performed. Three point mutations were identified by chemical mismatch cleavage, sequenced by dideoxynucleotide chain-termination sequencing and confirmed by allele-specific oligonucleotide hybridization of the patients' genomic DNA. The mutations resulted in the substitution of a tryptophan for an arginine at codon 1319, changing a hydrophobic to a basic hydrophilic amino acid, the substitution of a cysteine for a tyrosine at codon 1542, potentially losing a disulfide bond, and the substitution of a serine for a leucine at codon 1237. These are the first point mutations to be documented in the STS gene and may allow insight into functionally important domains of the protein.

Introduction

X-linked ichthyosis (XLI; MIM 30810) is an inborn error of metabolism caused by steroid sulfatase (STS) deficiency. It affects between one in 2,000 and one in 6,000 males, with a constant prevalence in a variety of geographical locations and racial groups. Clinically, it is characterized by extremely scaly skin and by corneal opacities not affecting visual acuity. The striking skin changes are seen at birth or within the first 4 mo of life, and consist of thick, adherent dark scales on the limbs and trunk, usually sparing palms and soles. These reflect a disorder of lipid metabolism in the epidermis caused by the deficiency of STS enzyme activity. A deficiency of placental STS results in low estriol levels in urine and plasma of the mothers of affected fetuses. This presumably is the cause of the delay of onset of labor, which is relatively unresponsive to oxytocic agents, and of the increased incidence of stillbirth (see Shapiro [1989] for review).

Steroid sulfatase is a microsomal enzyme expressed in essentially all tissues and hydrolyses various 3 beta-hydroxysteroid sulfates. The gene encoding STS, localized on the distal short arm of the X chromosome at Xp22.3 (Tiepolo et al. 1980), has been cloned (Ballabio et al. 1987; Bonifas et al. 1987; Conary et al. 1987; Yen et al. 1987). Studies on large numbers of ethnically diverse patients with XLI have shown that in more than 80% of cases the enzyme deficiency is due to large deletions involving the entire STS gene and flanking sequences (Ballabio et al. 1989; Shapiro et al. 1989). However, rare patients have been identi-

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fied who lack both enzyme activity and detectable cross-reacting material with STS antibodies but who show a normal restriction digest pattern when probed with the full-length STS cDNA (Wirth et al. 1988; Ballabio et al. 1989; Shapiro et al. 1989). We have studied five unrelated patients who fall into this group, using PCR on reverse-transcribed (RT) RNA and chemical mismatch cleavage to scan for point mutations.

Material and Methods

Patients

Patients were selected who exhibited typical clinical features of XLI and total absence of detectable STS activity in cultured skin fibroblasts. STS activity was assayed as described in a previous study (Ballabio et al. 1985). Five patients were studied: patient A (T.R.) was Scottish; patients B (C.C.), C (R.E.), D (R.B.), and E (A.O.) were Italian. Two of the patients, A and B, had been partially studied in a previous report (Ballabio et al. 1989).

Western Blot Analysis

Immunoblotting experiments were carried out on fibroblast glycoprotein preparation (Parenti et al. 1987) using anti-STS polyclonal antibodies (van der Loos et al. 1984).

RNA Isolation and cDNA Synthesis

Total RNA was isolated from cultured fibroblasts by both the guanidine isothiocyanate procedure (Chirgwin et al. 1979) and the procedure described below. Ten micrograms of total fibroblast RNA and 1 μg of a specific primer (5′-AGTGCCACTCTCAGGCGTGT-3′), located within the 3′ untranslated region (bp 2047–2028, according to Yen et al. [1987]) of the STS gene were mixed in 25 μl of 50 mM Tris HCl pH 8.3, 8 mM $MgCl_2$, 30 mM KCl and heated to 65°C for 5 min. The mixture was chilled on ice, then combined with 14 units of ribonuclease inhibitor (RNAsin; Pharmacia), 3 μl of 100 mM DTT, 1.5 μl of a 25 mM mixture of all four deoxyribonucleotide triphosphates (dNTPs), and 1.5 μl of Moloney murine leukemia virus reverse transcriptase (200 units/μl; BRL). Reactions were incubated at 37°C for 30 min. After 30 min, an additional 1.5 μl of reverse transcriptase were added, and the reaction continued for another 30 min.

Amplification of cDNA by PCR

The products of the cDNA synthesis from 10 μg of total fibroblast RNA were dissolved in 20 μl of water, and 5 μl of this were subjected to 30 cycles of PCR amplification (Mullis and Faloona 1987). The reaction volume was 50 μl; this included 10 μl of 5× Cetus buffer, 0.5 μl of 25 mM dNTPs, 0.5 μl *Taq* polymerase (5 μg/μl), and 400 ng of each primer, as described below. A total of five different amplification units were required to obtain full-length amplification of the cDNA. Amplification of the 422-bp segment required two rounds of sequential PCR. The oligonucleotide primers used in the PCR reactions have been numbered from 1 to 12 and their sequence is shown in table 1. Primers for the first round of the 422-bp segment were 1 and 2; for the second nested round of the 422-bp segment, 3 and 4; for the 209-bp segment, 5 and 2; for the 653-bp segment, 6 and 7; for the 454-bp segment, 8 and 9; and for the 539-bp segment, 10 and 11.

Chemical Mismatch Cleavage

Chemical cleavage reactions were carried out as originally described by Cotton et al. (1988, 1989) and as modified by Grompe et al. (1989, 1991). PCR products from a normal male served as probe. In a first amplification, stocks of wild-type PCR products for all four amplification units (see fig. 1) were generated, purified with a Centricon 100 column (Amicon), and frozen at a concentration of 1 ng/μl. In a next step,

Table 1

Sequences of Oligonucleotide Primers Used in PCR Analysis

Primer[a]	Sequence	Location
1 (F) ...	GCAATCTCCTCCATCACAGCTCAG	154–177
2 (R)...	GCTGTGACAGCTCATCCCAAGGTG	635–612
3 (F) ...	CAACAACAGGATCACAAGCTGGAG	183–206
4 (R)...	CCCTATCAGTGCTGTTGAATAACC	605–582
5 (F) ...	CTGTGCACACCAAGCAGGGCAGC	426–448
6 (F) ...	CTTCCCACCGATGAGATTACCTTTG	537–561
7 (R)...	CTCATCCAGAAGGTTCAAGATCTG	1190–1167
8 (F) ...	CACAGCCCTGTTCTCCAGCAAAGAC	1076–1100
9 (R)...	GAAACTCATGATCGGAGCGTTGGC	1530–1507
10 (F) ...	AGGACAGGATCATTGATGGACGTG	1456–1479
11 (R)...	CTTTGCCACATGCGTCTGTCTGG	1993–1971
12 (R)...	CATTTCTCACCTTTATAGATCCC	Intron–1290
13 (F) ...	TCCCCTCCAGGGCAGATCTTGAAC	Intron–1178
14 (R)...	CAGAGTACCTGTCCTCAGGCAAGG	Intron–1447
15 (F) ...	GATCTTTTAGGAGGAAAAGCAAAC	Intron–1316
16 (F) ...	TATCCCACAGGATCATTGATGGAC	Intron–1476
17 (R)...	TTTACTCACTGTTCTGAGGTGC	Intron–1571

NOTE.—The sequence location of the primers is numbered according to Yen et al. (1987). Primers 12–17 are across a splice site (Yen et al. 1988).

[a] F = forward; and R = reverse.

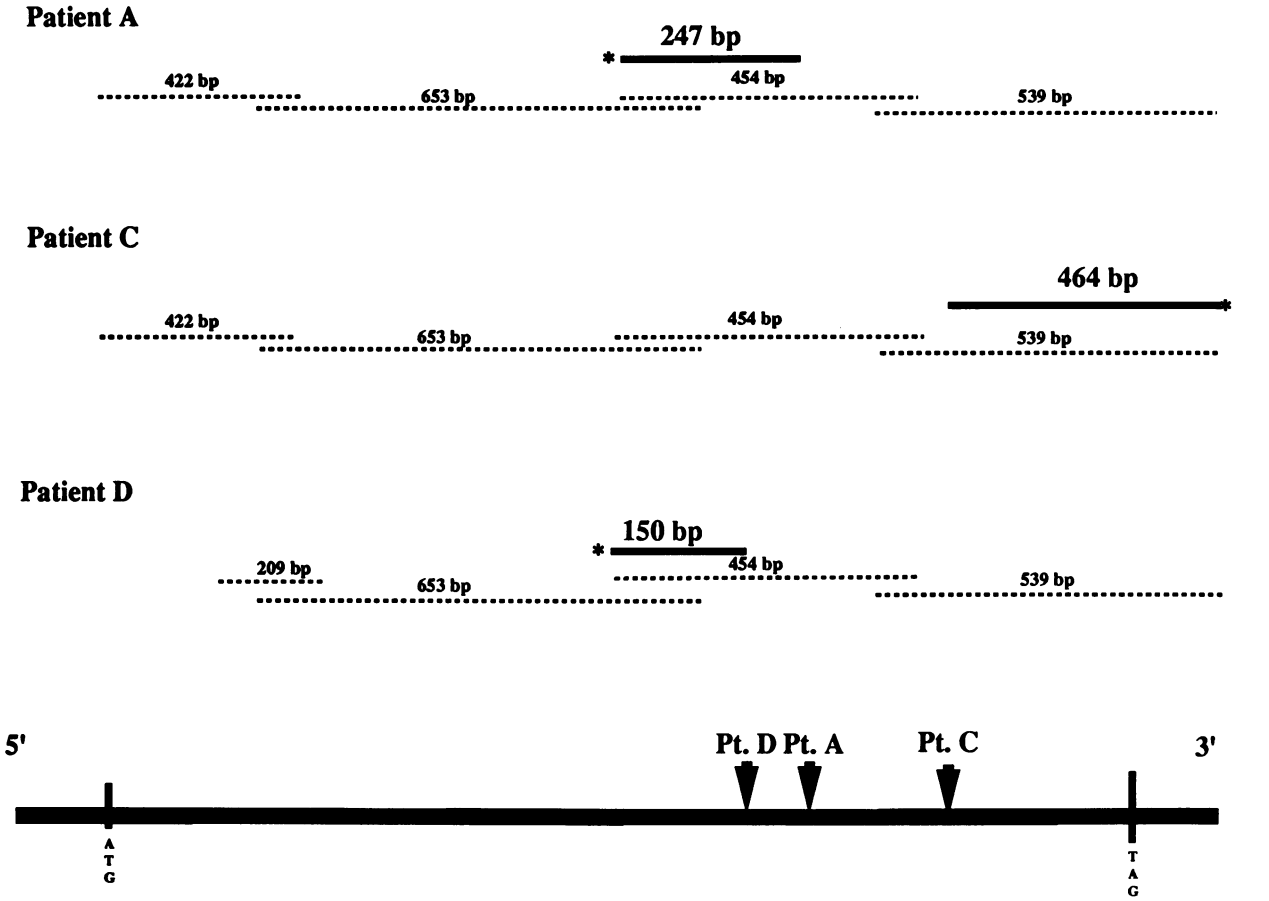

Figure 1 Schematic representation of mismatch sites and cleavage fragments in STS cDNA. The position of the cleavage products (solid lines) are aligned with the PCR amplification products (dashed lines) and with the STS cDNA. The arrows indicate the predicted site of each point mutation, based on the size of the cleavage product. Note that patient D had a different PCR amplification unit from the other two patients and did not amplify at the 5' end.

each of the primers used in the amplification of the STS cDNA was individually end labeled by a kinase reaction as described by Sambrook et al. (1989). After the reaction the volume was then brought up to 1 ml with water, and the labeled oligonucleotide was cleaned up and concentrated to 40 µl by centrifugation through a Centricon 3 column (Amicon). One nanogram of the stock of wild-type PCR product was then reamplified in eight separate reactions each employing one labeled and the corresponding unlabeled primer. The reamplifications resulted in strand-specific radiolabeled PCR probes for all four amplification units of the STS cDNA. The amplification conditions were identical to those used for the PCR of cDNA except for the reaction volume and the primer concentrations. The total reaction volume was 60 µl, and 3 pmol of both the labeled and unlabeled primer was used. After amplification the probes were purified in a 1% low-melting-temperature agarose gel and extracted by electroelution into a Centricon 100 column. The concentration was estimated by comparison to standard size markers in an ethidium bromide–stained agarose gel and adjusted to ~10 ng/µl (~50,000 cpm). PCR amplification was also carried out on cDNA from patients as described above. The products were purified by centrifugation through a Centricon 100, and the concentration was adjusted to 50 ng/µl. For the heteroduplex formation 6 µl of 5 × annealing (3M NaCl, 30 mM Tris/HCl buffer pH 7.7, 35 mM $MgCl_2$), 150 ng of cold target (mutant PCR product), and 10 ng of the wild-type probe were mixed, and the volume was adjusted to 30 µl. This represents a 15-fold molar excess of the mutation-containing DNA. The mixture was then heated in a 1.5-ml tube in a boiling water bath for 5 min. The droplets were then spun down, and the tubes were transferred to a 42°C water bath

for 2 h. Then 90 μl of ice-cold ethanol were added, and the DNA was precipitated. The precipitated heteroduplex was redissolved in 20 μl H_2O, and 6 μl of this was used for the subsequent chemical modifications. All reactions were carried out in siliconized 1.5-ml tubes. Twenty microliters of hydroxylamine solution (1.39 g hydroxylamine chloride in 1.6 ml H_2O, buffered to pH 6.0 with diethylamine) was mixed with the heteroduplex and then incubated at 37°C for 20 min. For the osmium tetroxide reaction 2.5 μl of 10 × osmium buffer (100 mM Tris/HCl pH 7.7, 10 mM EDTA, and 15% pyridine) were added to each heteroduplex. The tubes were placed on ice, and 1 μl of 2% osmium tetroxide (aqueous solution; Aldrich) was mixed in thoroughly by pipetting up and down. Then a 5-min incubation at 37°C was carried out. Both the osmium tetroxide and the hydroxylamine reactions were stopped by the addition of 200 μl of stop buffer (0.3 M sodium acetate pH 5.2, 0.5 mM EDTA, and 25 μg baker's yeast tRNA/ml). The reaction mix was then precipitated with 750 μl of ethanol, washed once with 75% (v/v) ethanol/water, and dried. The dried pellet was then resuspended in 50 μl of 1 M piperidine and incubated at 90°C for 30 min. Then an equal volume of 0.6 M sodium acetate pH 5.2 and 300 μl ethanol were added for precipitation. After a final wash with 75% (v/v) ethanol/water the reactions were dried and redissolved in 15 μl formamide loading buffer. Three to six microliters was then electrophoresed in a 4% wedged denaturing polyacrylamide gel. The gels were then fixed with methanol:acetic acid:water at 1:1:8 and dried, and a Kodak X-ray film was exposed for 24 h.

Sequencing

The PCR products of interest were subcloned into vector PTZ19U (Pharmacia). Dideoxy chain-termination (Sanger et al. 1977) sequencing was performed on single-strand templates (Vieira and Messing 1987) employing fluorescent sequencing primers and an Applied Biosystem ABI 370A fluorescent sequencer (Gibbs et al. 1989).

Amplification of Genomic DNA by PCR and Allele-specific Oligonucleotide (ASO) Hybridization

Primers flanking the STS gene of interest were used for a 30-cycle PCR amplification of approximately 1 μg of genomic DNA isolated from peripheral white blood cells by standard techniques (Sambrook et al. 1989). The buffers and concentrations of the reagents were identical to those used for the cDNA amplification described above. Amplification conditions were initial denaturing at 94°C for 7 min, 30 cycles of denaturing at 90°C for 30 s, hybridizing at 50°C for 30 s, and polymerase extension at 70°C for 1.5 min. Oligonucleotide primers have been numbered from 12 to 17, and their sequences are shown in table 1. Primers flanking exon 7 are 12 and 13, flanking exon 8 are 14 and 15, and flanking exon 9 are 16 and 17.

After amplification of the appropriate exons from genomic DNA as described above, 20 μl of the PCR reaction mixture was alkaline denatured in 0.4 M NaOH, 25 mM EDTA and blotted onto a Gene Screen Plus (Dupont) membrane via a slot blot apparatus (Schleicher & Schuell). Six 19-base oligonucleotides were synthesized, two for each patient, corresponding to either the wild-type sequence or the same sequence except for the base at position 10, which was specific for the point mutation. ASOs were radiolabeled and hybridized to the filter (Gene Screen Plus, NEN-Dupont) at 37°C in 6 × SSPE, 1% SDS for 2 h. The filters were washed (2 × SSC, 0.5% SDS) at a variety of temperatures and autoradiographed for 30 min at room temperature.

Results

Five unrelated patients with typical XLI phenotypes were studied. All had complete lack of STS activity when assayed enzymatically (data not shown). Western blot analysis was carried out on total protein extracts from their fibroblasts. No cross-reacting material was detected when the filter was probed by anti-STS antibodies (fig. 2). Southern blotting of genomic DNA using full-length STS cDNA as a probe detected no alterations in the gene (data not shown). We elected to scan for mutations in the coding region.

Fibroblast cell cultures were grown, total RNA was isolated, and first-strand cDNA was synthesized by reverse transcription. The STS cDNA was amplified by PCR in multiple fragments (fig. 1). No differences in the molecular weight of the wild-type and mutant fragments were detected by PAGE of those fragments that amplified. The 422-bp 5′ fragments of both the wild type and mutants required nested primers to produce a detectable signal. Three of the patients, A, C, and E, amplified along the entire cDNA. Patient D amplified only a 209-bp portion of the 422-bp 5′ fragment but amplified the remainder of the cDNA. Patient B amplified no part of the cDNA except the 422-bp 5′ fragment. Northern blot analysis was un-

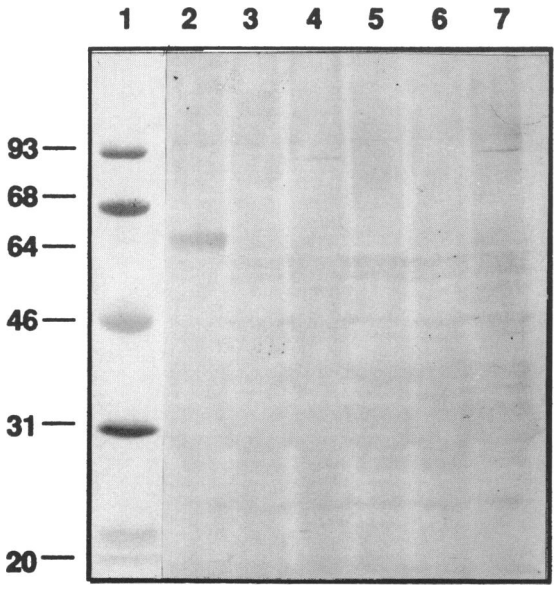

Figure 2 Western blot analysis of fibroblast protein from XLI patients, probed with antibody specific for STS. Cross-reacting material is absent in patients B–E. Lane 1, molecular weight standards (in kilodaltons). Lane 2, Wild-type control with the STS band at 64 kDa clearly visible. Lane 3, XLI patient with total deletion of STS gene. Lane 4, Patient C. Lane 5, Patient D. Lane 6, Patient B. Lane 7, Patient E.

successful, presumably owing to extremely low levels of mutant STS polyA mRNA.

A strand-specific wild-type probe was generated by PCR amplification using only one radioactive primer for each of the PCR units spanning the cDNA. The probe was then used in the formation of a heteroduplex with the patient's corresponding PCR unit. Chemical modification and piperidine cleavage were performed on all the PCR units, including those from patients whose units did not cover the entire cDNA. All regions were tested by both osmium and hydroxylamine, and each with labeled primers from both the 5′ and 3′ ends. The results of the chemical cleavage analysis is shown in figure 3 and diagrammed in figure 1. Point mutations were found in patients A, C, and D. Patient A showed a mismatch site ~250 bp from the 5′ end of the 454-bp PCR fragment when cleaved with osmium tetroxide using the sense probe, indicating that a thymidine is altered. Patient C had a mismatch site ~460 bp from the 3′ end of the 539-bp fragment when cleaved with hydroxylamine using the antisense probe. This indicated an altered cytosine, so that the mutant would lack a guanosine. Patient D had a mismatch site ~150 bp from the 5′ end of the 454-bp fragment when cleaved with hydroxylamine using the sense probe. Thus, this patient had an altered cytosine. The other amplified mutant PCR fragments did not yield any cleavage products, suggesting that point mutations within the amplified regions are not the basis of their lack of STS. In addition to the putative sites of mutation found in these three patients, two other patients, one with complete and the other with partial amplification of the cDNA, failed to yield any mismatch cleavage products.

The three mismatch sites were sequenced after cloning of the PCR products into PTZ19U and correlated well with the chemical mismatch cleavage predictions. The sequence alterations are summarized in table 2.

In order to verify the presence of the point mutations in the patients' genomic DNA, ASO studies were performed by using 19-bp oligonucleotides. The exon containing the point mutation was amplified by PCR from both patient and wild type. The amplification products were dot blotted onto nitrocellulose filters and probed with either a 19-base ASO specific for the wild-type sequence or an ASO corresponding to the same sequence except for the base at position 10, which was specific for the point mutation. These results are shown in figure 4 and confirm the mutation in each case. Although the mutant probe has a clear affinity for patient A as compared to wild-type DNA, the wild-type probe did not differ in its melting temperature with the two different DNAs.

To investigate the likelihood of polymorphism versus true mutation, similar studies were performed by using ASOs from patient D to probe genomic DNA from 22 unrelated females (44 X chromosomes). The mutant probe hybridized only to the patient's DNA, and failed to hybridize to any of the normal controls (fig. 5). Similar results were obtained in patient A (data not shown). Normal DNA specimens were not surveyed for patient C's mutation.

Discussion

The molecular basis of STS deficiency leading to the clinical picture of XLI is of particular interest because of the unusually high frequency of total gene deletions. This has hampered studies of gene regulation as well as functional studies of the STS protein because of the rarity of other types of mutations. To date, only a few patients retaining STS X-chromosome-encoded sequences have been reported (Wirth et al. 1988; Ballabio et al. 1989; Shapiro et al. 1989). Two of these patients have had partial deletions: one had a deletion

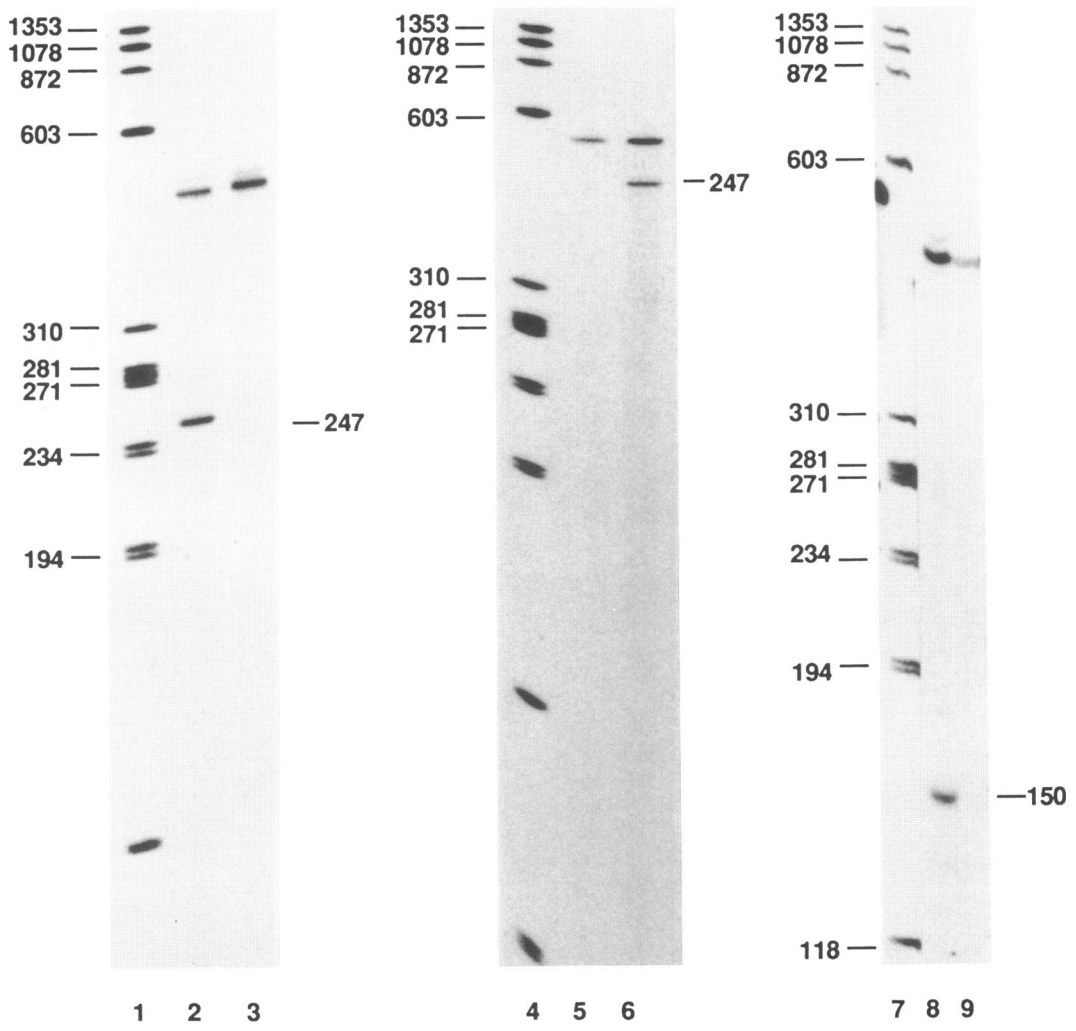

Figure 3 Chemical mismatch cleavage of STS cDNA in three XLI patients. The agent used for the modification reaction is indicated above each lane (OS = osmium tetroxide; and H = hydroxylamine). The radiolabeled PCR primer used to prepare each probe, numbered to correspond to those listed in table 1, also appears above each lane. Lanes 2, 6, and 8 contain cleavage products from patients A, C, and D, respectively. Lanes 3, 5, and 9 contain DNA from patients that did not cleave, and thus serve as negative controls. Molecular weight standards are in lanes 1, 4, and 7.

Table 2

Codon Changes

Patient	Codon Change		Amino Acid Change	Predicted Alteration
	Position[a]	Change		
A	1320	TGG→AGG	Trp→Arg	T, codons 1316–1330
C	1543	TGC→TAC	Cys→Tyr	G, codons 1516–1544
D	1226	TCG→TTG	Ser→Leu	C, codons 1228–1234

[a] Codon numbering system according to Yen et al. (1987).

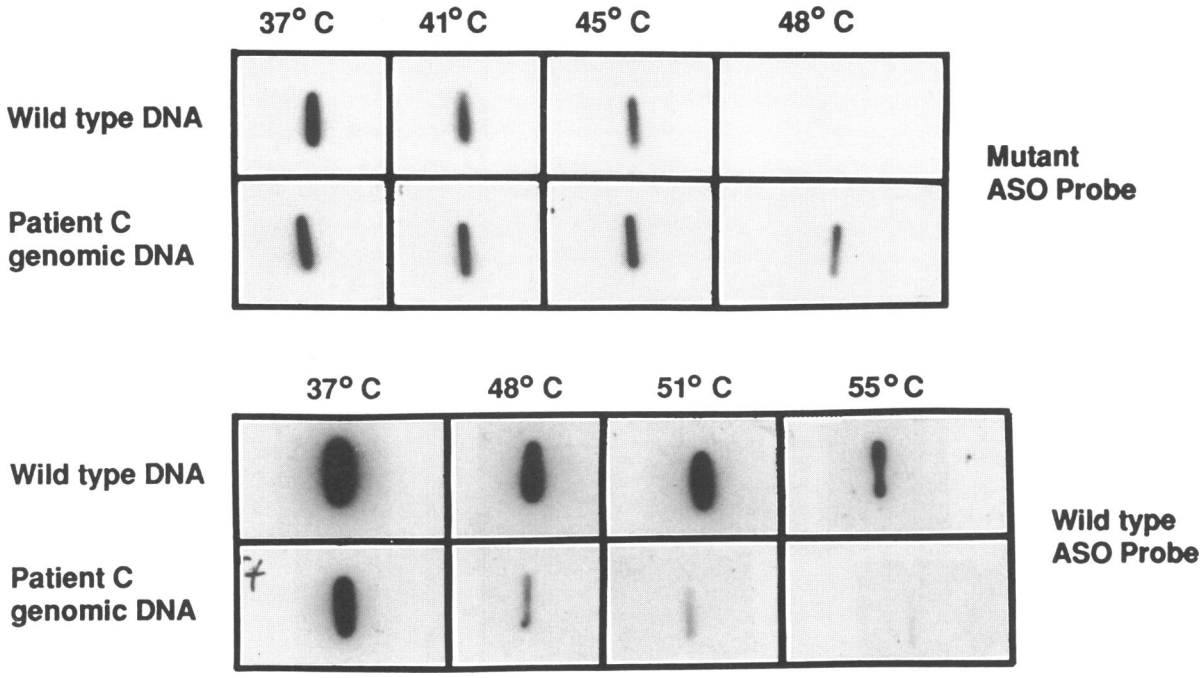

Figure 4 Confirmation of patient C point mutation in genomic DNA by ASO hybridization. Mutant and wild-type ASO hybridization was carried out on the PCR-amplified STS gene exon 9 (with primers 17 and 18 as listed in table 1), containing the predicted mutation from both wild-type and patient C STS cDNA. The amplification products were dot blotted onto nitrocellulose filters and probed with either a 19-base ASO specific for the wild-type sequence (lower pair of blots) or an ASO corresponding to the same sequence except for the base at position 10, which was specific for the point mutation (upper pair of blots). The upper row of each pair of test blots contains wild-type DNA, and the lower row contains DNA from patient C. Washing temperatures are indicated above each column.

at the 3′ end of the gene (Ballabio et al. 1989) and the other lacked exons 2–5 (Shapiro et al. 1989). All the others show a normal pattern when hybridized to the full-length STS cDNA.

The three point mutations reported in this study are the first identified in the STS gene. According to the chemical mismatch cleavage data, two of the patients having point mutations (A and C) had no other devia-

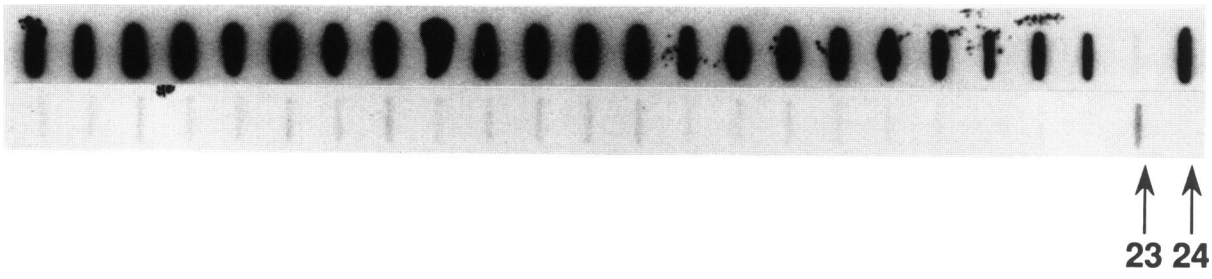

Figure 5 Polymorphism study of point mutation from patient D, by ASO confirmation. STS gene exon 7 was PCR amplified from total genomic DNA with primers 13 and 14, as numbered in table 1. Lanes 1–22, STS gene exon 7 amplified DNA from 22 unrelated normal females. Lane 23, DNA from patient D. Lane 24, DNA from a normal male control. The upper blot was hybridized with a 19-base ASO specific for the wild-type sequence. The lower blot was hybridized with an ASO corresponding to the same sequence except for the base at position 10, which was specific for the point mutation. The upper blot was washed at 51°C, the lower at 48°C. Under these conditions, the wild-type probe hybridizes with all but the patient's DNA, and the mutant probe hybridizes with only the patient. This verifies the presence of the point mutation at the genomic level and suggests that it is not a polymorphism, since it is not present in any of the normal X chromosomes investigated.

tion from the wild-type sequence along the entire coding region. The presence of additional sequence alterations, not detected by chemical mismatch cleavage, is very unlikely, since the accuracy of the methods has been demonstrated to exceed 98% (Cotton 1990). The method has also been used for the detection of point mutations in other genetic diseases, such as osteogenesis imperfecta (Lamande et al. 1989), ornithine transcarbamoylase (OTC) deficiency (Grompe et al. 1989), and phenylketonuria (Dianzani et al. 1991), and its reliability has been found to be excellent. Patient D, however, may have an additional abnormality at the far 5′ end of the coding region that could not be detected because this area failed to amplify with PCR. Our method would not be able to detect some mutations at the splice sites or in the promoter region; these mutations could therefore be present in the patients in whom no mutations were found. ASO hybridization using both the wild-type and the mutant oligo confirmed the mutations in the patient's genomic DNA and ruled out PCR-derived mutations. Additional ASO studies were done for two of the mutations to rule out polymorphisms in the normal population, and the same mutations were not found in 44 X chromosomes from normal individuals studied. These oligos can now be used to screen other nondeletion cases with STS deficiency to identify possible mutation hot spots.

Each of the point mutations reported here resulted in an amino acid change of theoretically functional significance. These changes are summarized in table 2. Patient A had a substitution of tryptophan to arginine, changing a hydrophobic to a basic hydrophilic amino acid. The mutation in patient C changed a cysteine to tyrosine, potentially destroying a disulfide bond. Patient D had a serine replaced by leucine. None of these mutations affect amino acids found to be conserved in all the characterized human arylsulfatases (arylsulfatase A, arylsulfatase B, and steroid sulfatase) (Schuchman et al. 1990).

All the "nondeletion" patients studied, including the five patients described here, showed absence of STS cross-reacting material at the immunoblotting analysis. The mutations described in this study may, therefore, result in an unstable STS protein that may be rapidly degraded. Alternatively, the absence of STS cross-reacting material may be due to messenger RNA instability, as suggested by the difficulty encountered in amplifying the patients' reverse-transcribed RNA with respect to that of normal individuals. Although the finding of RNA instability in missense mutations is not common, it has already been reported in a patient with OTC deficiency (Grompe et al. 1989) and may be due to an altered secondary structure of the RNA. Lastly, the mutations described may affect STS epitopes, which are important for the binding of the antibodies. Although in vitro expression studies will be needed to demonstrate the functional significance of these sequence alterations, we have identified three potentially important regions of the STS protein.

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